ACTIVATION OF G-PROTEINS BY RECEPTOR-STIMULATED NUCLEOSIDE DIPHOSPHATE KINASE IN DICTYOSTELIUM

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Activation of G-proteins by receptor-stimulated nucleoside diphosphate kinase in *Dictyostelium*

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Recently, interest in the enzyme nucleoside diphosphate kinase (EC 2.7.4.6) has increased as a result of its possible involvement in cell proliferation and development. Since NDP kinase is one of the major sources of GTP in cells, it has been suggested that the effects of an altered NDP kinase activity on cellular processes might be the result of altered transmembrane signal transduction via guanine nucleotide-binding proteins (G-proteins). In the cellular slime mould *Dictyostelium discoideum*, extracellular cAMP induces an increase of phospholipase C activity via a surface cAMP receptor and G-proteins. In this paper it is demonstrated that part of the cellular NDP kinase is associated with the membrane and stimulated by cell surface cAMP receptors. The GTP produced by the action of NDP kinase is capable of activating G-proteins as monitored by altered G-protein-receptor interaction and the activation of the effector enzyme phospholipase C. Furthermore, specific monoclonal antibodies inhibit the effect of NDP kinase on G-protein activation. These results suggest that receptor-stimulated NDP kinase contributes to the mediation of hormone action by producing GTP for the activation of GTP-binding proteins

Key words: Dictyostelium discoideum/G-proteins/NDP kinase/phospholipase C/surface receptors

Introduction

Nucleoside diphosphate kinase (NDP kinase) has been known for many years as a major source for all nucleoside triphosphates except ATP. The enzyme catalyses the transfer of a high energy phosphate from a nucleoside triphosphate, generally ATP, to a nucleoside diphosphate. The reaction is a so-called ping-pong reaction involving a high energy phosphohistidine intermediate (Parks and Agarwal, 1973).

Renewed interest in this enzyme derives from its identification as the product of the *awd* gene from *Drosophila*, mutations of which cause abnormal development and larval death (Dearolf *et al.*, 1988a,b; Rosengard *et al.*, 1989; Biggs *et al.*, 1990). NDP kinases are also encoded from the human genes *Nm23-H1* and *Nm23-H2* (Gilles *et al.*, 1991), which are involved in cell proliferation (Hailat *et al.*, 1991; Keim *et al.*, 1992). *Nm23-H1* levels are decreased in highly metastatic cell lines (Leone *et al.*, 1991), but its link with the metastatic status of tumours remains to be established (Lacombe *et al.*, 1991; Sastre-Gareau *et al.*, 1992). NDP kinase may physically interact with G-proteins (Nickerson and Wells, 1984; Ohtsuki *et al.*, 1985, 1986; Wieland *et al.*, 1986; Kimura and Shimada, 1988; Lacombe and Jakobs, 1992). The NDP kinase from *Dictyostelium discoideum* (Lacombe *et al.*, 1990) is highly homologous to its counterparts in higher eukaryotes (Wallet et al, 1990; Gilles *et al.*, 1991).

Dictyostelium cells use extracellular cAMP as chemoattractant and morphogen. cAMP is detected by surface receptors and the signal is transduced by one or more G-proteins to adenylyl cyclase, guanylyl cyclase and phospholipase C (Van Haastert *et al.*, 1991). Incubation of *D.discoideum* membranes with the non-hydrolysable GTP analogue GTPγS results in a reduced affinity of the receptor for its ligand cAMP, due to the activation of the associated G-protein (Van Haastert, 1984; Snaar-Jagalska and Van Haastert, 1988).

In this report we show that *Dictyostelium* membranes possess a cAMP surface receptor-stimulated NDP kinase that produces GTP from exogenous GDP. This reaction, which leads to the activation of G-proteins and phospholipase C, is inhibited by monoclonal anti-NDP kinase antibodies.

Results

Identification of NDP kinase

To establish whether membranes from starved *Dictyostelium* cells contain NDP kinase, crude membranes containing membranes, nuclei and other organelles were incubated with [γ^{32}P]ATP. Two major acid and heat labile phosphoproteins were observed with molecular weights of 20 and 36 kDa (Figure 1). An antibody directed against *Dictyostelium* NDP kinase is able to specifically immunoprecipitate p20, identifying p20 as a subunit of NDP kinase. The incorporation of radioactivity from ATP into p36 is stimulated by guanine nucleotides and inhibited by adenine nucleotides, succinate and co-enzyme A (data not shown), indicating that p36 is the α-subunit of succinate synthetase; the labelling of this protein has been observed previously in *Dictyostelium* (Van Haastert, 1987).

Receptor stimulation of NDP kinase-mediated GTP production

Incubation of *Dictyostelium* membranes with Mg^{2+}-[^{32}P]ATP leads to the formation of [^{32}P]GTP. This activity can be stimulated ~2-fold by the receptor agonist cAMP (Figure 2 and inset). Since GDP was not added to the membranes, NDP kinase-mediated phosphorylation must have used endogenous GDP. cAMP may stimulate GTP formation by activating NDP kinase or by providing more GDP (e.g. by releasing bound GDP from G-proteins, tubulin

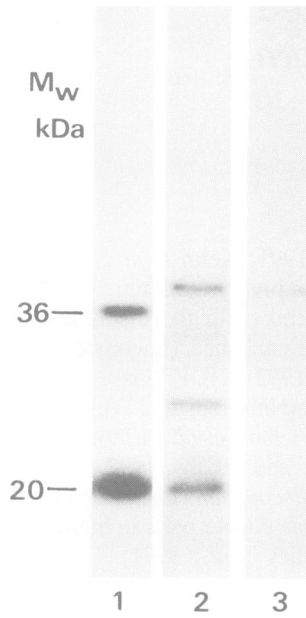
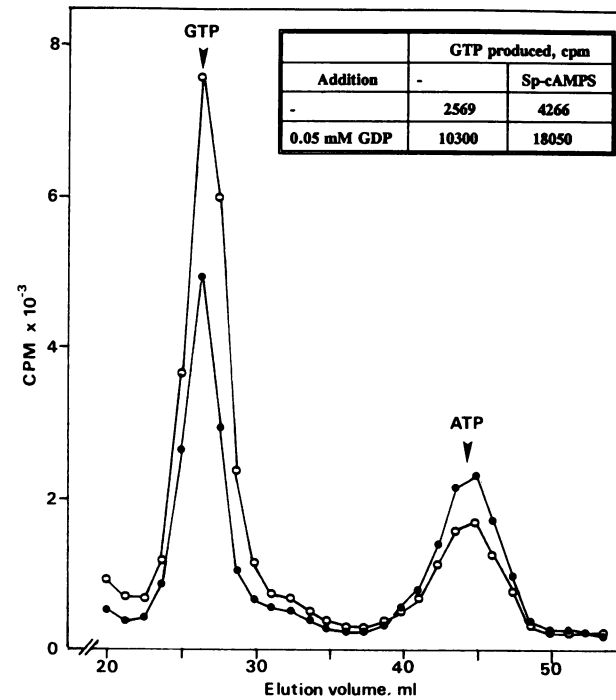


Fig. 1. NDP kinase in *Dictyostelium* membranes. Lane 1, crude membranes were incubated with [^{32}P]ATP for 10 min and the protein-associated radioactivity was analysed by SDS−PAGE; lane 2, immunoprecipitate using a polyclonal anti-NDP kinase antibody; lane 3, immunoprecipitate obtained with preimmune serum. Lane 1 was exposed for 16 h, whereas lanes 2 and 3 were exposed for 48 h. Different exposure times are needed because a substantial amount of NDP kinase-associated radioactivity is lost during the immunoprecipitation procedure as a result of the instability of the histidine phosphate in NDP kinase.



Addition	GTP produced, cpm	
	-	Sp-cAMPS
-	2569	4266
0.05 mM GDP	10300	18050

Fig. 2. Receptor-stimulated NDP kinase activity. HPLC profile of the produced [^{32}P]GTP from GDP and [^{32}P]ATP following the incubation of membranes with [^{32}P]ATP in the presence (●) or absence (○) of the receptor agonist cAMP. *Inset*, c.p.m. GTP obtained under various conditions.

etc.). To exclude this latter possibility, 50 μM GDP was added to the membranes so that GDP could no longer be a limiting factor in the reaction. Under these conditions, incubation with Mg^{2+}−[^{32}P]ATP leads to the increased formation of [^{32}P]GTP compared with the situation without added GDP, however, GTP production is still further stimulated by cAMP (Figure 2, inset) indicating that the enhancement of GTP production by cAMP is due to an increase of the activity of NDP kinase and not solely to the release of bound GDP. UDP or other nucleoside diphosphates give analogous results. Incubation of membranes with Mg^{2+}−[^{35}S]ATPγS leads to the formation of [^{35}S]GTPγS by NDP kinase and this reaction is also stimulated by cAMP (data not shown). These results imply that *Dictyostelium* membranes possess a cAMP surface receptor-mediated activation of NDP kinase.

G-protein activation by NDP kinase derived GTPγS

Activation of G-proteins by GTPγS has been shown to reduce the affinity of the receptor for agonist and to activate phospholipase C. As mentioned previously, GTPγS is efficiently synthesized by NDP kinase from ATPγS and GDP. Figure 3 shows that incubation of *Dictyostelium* membranes with Mg^{2+}−ATPγS leads to a reduction of cAMP binding to surface receptors and this effect is enhanced by the cAMP analogue adenosine 3′,5′-monophosphorothioate, Sp isomer (Sp-cAMPS) (Figure 3A). This reduction in cAMP binding is due to a decreased affinity for cAMP (see also Van Haastert, 1987). The role of the surface receptor in modulating cAMP binding by Mg^{2+}−ATPγS was investigated by preincubating membranes with Mg^{2+}−ATPγS and different concentrations of cAMP analogues. The order of decreasing binding affinity of the analogues for the surface receptor is cAMP > 2′deoxy-cAMP > Sp-cAMPS >> 8Br-cAMP, and for cAMP-dependent protein kinase 8Br-cAMP > cAMP > Sp-cAMPS >> 2′deoxy-cAMP (Van Ments-Cohen and Van Haastert, 1989). The results presented in Figure 3B demonstrate that cAMP potentiates the effect of Mg^{2+}−ATPγS with a cyclic nucleotide specificity identical to that of the surface receptor and very different from cAMP-dependent protein kinase, indicating that the effect of cAMP is mediated directly by the cAMP surface receptor and not via protein kinase A.

Activation of G-proteins not only leads to a reduced receptor affinity for cAMP, but also stimulates effector enzymes such as phospholipase C. As shown in Figure 3C, incubation of a cell lysate with Mg^{2+}−ATPγS leads to the activation of phospholipase C, and this reaction is also stimulated by the receptor agonist cAMP.

The previous experiments suggest that ATPγS is used by a receptor-stimulated NDP kinase to generate GTPγS, which in turn activates a G-protein. To investigate this reaction further, two monoclonal antibodies against *Dictyostelium* NDP kinase were used that both recognize NDP kinase with high affinity ($K_D \sim 10^{-9}$ M). While mAb8-6 neutralizes NDP kinase activity, mAb9-7 does not (Figure 4A). Membranes were preincubated with the monoclonal antibodies, further incubated with Mg^{2+}−ATPγS, washed and assayed for binding of cAMP to surface receptors (Figure 4B). Only the neutralizing antibody mAb8-6 counteracts the Mg^{2+}−ATPγS induced decrease of cAMP binding, whereas incubation in the presence of non-neutralizing antibodies has no effect. These results clearly show that the decrease in affinity of the surface receptors is due to NDP kinase activity. None of the antibodies used

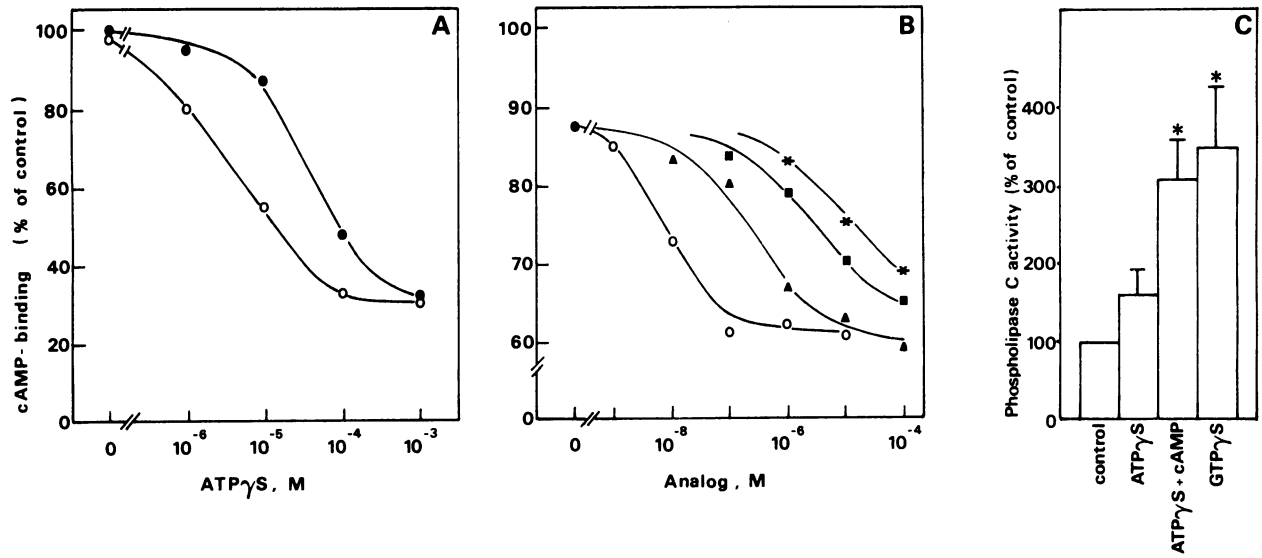


Fig. 3. Activation of G-proteins by NDP kinase. The activation of G-proteins was measured indirectly by analysing the effects of Mg^{2+}–ATPγS on agonist binding to surface receptors. (**A**) With (○) or without 10^{-5} M Sp-cAMPS (●). (**B**) 10^{-6} M ATPγS and cAMP (○), 2′deoxy-cAMP (▲), Sp-cAMPS (■) and 8-Br-cAMP (*). (**C**) Activity of phospholipase C in the presence of 10^{-6} M ATPγS, 10^{-6} M ATPγS + 10^{-6} M cAMP or 10^{-5} M GTPγS. Data are expressed as a percentage of control (100% corresponds to 80 pmol/min/10^7 cells) and are the mean ± SEM of four experiments in duplicate. *, significantly different from control with $P<0.05$.

alter inhibition of cAMP binding when GTPγS is applied directly, indicating that the direct activation of G-protein by GTPγS and its interaction with the receptor are unaltered by the antibodies (Figure 4B). The effect of the antibodies on ATPγS-mediated activation of phospholipase C could not be measured, because the enzyme is stable for only 1 min, which is insufficient to neutralize NDP kinase activity.

Summarizing, our results demonstrate that *D.discoideum* membranes possess receptor-stimulated NDP kinase activity that phosphorylates endogenous GDP to GTP or GTPγS using exogenous ATP or ATPγS, respectively. The produced GTP or GTPγS stimulates one or more G-proteins leading to the altered affinity of the surface cAMP receptor and activation of phospholipase C.

Discussion

A putative role of NDP kinase in the activation of GTP-binding proteins is emerging, although attempts to resolve the biochemical mechanism underlying this role are still a source of controversion. NDP kinase co-purifies with several GTP-binding proteins (Nickerson and Wells, 1984; Ohtsuki *et al.*, 1985, 1986; Wieland *et al.*, 1986; Kimura and Shimada, 1988; Lacombe and Jacobs, 1992). In *Drosophila* the gene *Awd* involved in development encodes a NDP kinase (Biggs *et al.*, 1990). In this organism, the mutant *killer of prune*, displaying a conditional dominant lethal interaction with the prune mutation (Sturtevant, 1956), is an allele of *awd* (Biggs *et al.*, 1988) encoding a point mutant of NDP kinase with altered subunit interaction (Lascu *et al.*, 1992). Interestingly, the *prune* gene may be a *Drosophila* homologue to a GTPase activating protein (Theng *et al.*, 1992).

In this paper we show that NDP kinase activity is stimulated by surface receptors and that the GTP produced by NDP kinase is capable of activating G-proteins. These results provide new biochemical evidence for a possible involvement of NDP kinase in transmembrane signal transduction.

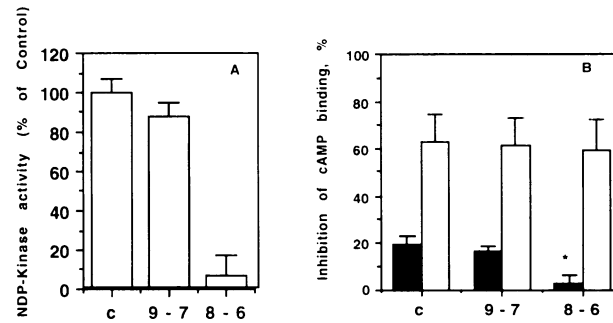


Fig. 4. Neutralization of NDP kinase by anti-NDP kinase monoclonal antibodies. (**A**) Inhibition of NDP kinase activity. Recombinant NDP kinase was incubated overnight without antibodies (C), with the mAb8-6 (8-6) or with mAb9-7 (9-7), both at 3 μg/ml. (**B**) Inhibition of ATPγS–Mg^{2+}-induced changes in receptor affinity. Membranes were prepared as described and preincubated for 20 min on ice with the antibodies at 10 μg/ml. Subsequently the membranes were incubated with 10 μM ATPγS and 5 mM $MgCl_2$ for 5 min at 20°C, washed and assayed for cAMP binding. The binding reaction was performed in the presence (open bars) or absence of μM 10 GTPγS (filled bars). Data are expressed as the percentage inhibition of cAMP binding ± SEM (mean of three independent experiments in triplicate); *, significantly different from control with $P < 0.05$.

Several mechanisms for the action of NDP kinase during transmembrane signal transduction have been proposed. The direct phosphorylation of bound GDP has been suggested for heterotrimeric G-proteins (Kikkawa *et al.*, 1990) and the small GTP-binding protein ARF (Randazzo *et al.*, 1991) but these conclusions have been retracted (Kikkawa *et al.*, 1991; Randazzo *et al.*, 1992). Recently the three-dimensional structure of NDP kinase was resolved (Dumas *et al.*, 1992). It also suggests that it is unlikely that *in situ trans*-phosphorylation of GDP bound to G-proteins can occur. Another mechanism could be that NDP kinase supplies an increased local concentration of GTP. However, under physiological conditions, cellular GTP concentrations seem sufficient for a normal exchange of GDP by GTP, raising the question of what could be the cellular function of NDP

kinase. A possibility is that NDP kinase is only active in highly dynamic processes demanding large amounts of GTP, such as cellular signalling during aggregation in *Dictyostelium*, or cell proliferation and differentiation in mammalian cells and *Drosophila*, respectively. A third possible mechanism is that NDP kinase, the G-protein and the receptor are in close contact and that *in vivo*, NDP kinase phosphorylates GDP as soon as it leaves the G-protein, thus shortening the time needed for reloading the G-protein. A single NDP kinase hexamer in a phosphorylated state could activate six G-proteins before it has to be reloaded thus NDP kinase could function as a local buffer of high energy phosphate. In mammalian cells, two polypeptides (NDPK-A and NDPK-B) can associate to form hybrid hexamers (Gilles *et al.*, 1991). An association of NDP kinase with a specific structure is also suggested by the fact that most of the sequence variation between the two types of NDP kinase is localized at the surface of the native hexameric enzyme, providing for possible specific interactions (Dumas *et al.*, 1992).

In conclusion, the present observation of GTP formation and the activation of GTP-binding proteins by receptor-stimulated NDP kinase allows for an additional level of regulation in signal transduction. It may also contribute to the activation of specific GTP-binding proteins including those that are not directly regulated by surface receptors, such as p21-RAS and other members of the family of small GTP-binding proteins.

Materials and methods

Materials

[γ-^{32}P]ATP (>3000 Ci/mmol) was from Amersham, EDTA, HEPES, 2′deoxy cAMP, 8-Bromo cAMP and cAMP were from Sigma. ATP, GDP, ATPγS, GTPγS, succinate and co-enzyme A were from Boehringer Mannheim. Sp-cAMPS was a kind gift of Dr B.Jastorf (University of Bremen, Germany). All other chemicals used were at least analytical grade.

Membrane preparation

Dictyostelium cells (strain NC4) were starved for 5 h in 10 mM KH_2PO_4–Na_2HPO_4, pH 6.5 (PB), washed and resuspended in 40 mM HEPES pH 7.0, 0.5 mM EDTA (HE buffer) at a density of 2×10^8 cells/ml. Cells were lysed by rapid elution through a nuclepore polycarbonate filter (pore size 3 μm), the lysate was centrifuged for 5 min at 10 000 *g*, the pellet was washed once and the final pellet was resuspended in HE to a density equivalent to 10^8 cells/ml.

Protein labelling and immunoprecipitation

The incubations (100 μl) contained 10 mM NaF, 0.5 μCi [γ-^{32}P]ATP in HE buffer and 40 μl crude membranes. After 10 min the reactions were terminated by the addition of sample buffer (Laemmli, 1970) and proteins were separated by SDS–PAGE. For immunoprecipitation sample buffer contained 25 mM Tris–HCl pH 6.8, 1.5% SDS, 5% glycerol and 0.25% dithiothreitol; the incubation with anti-NDP kinase serum and preimmune serum was for 2 h at a 1:500 dilution, followed by an incubation with 16 mg/ml protein A–Sepharose for 1 h. Sepharose-associated protein was separated by SDS–PAGE and dried gels were exposed to Kodak X-ray film.

GTP production and HPLC

Dictyostelium membranes were incubated at 22°C with 1 μCi [γ-^{32}P]ATP and 5 mM $MgCl_2$ in the presence or absence of 10 μM (Sp)cAMPS and 50 μM GDP, as indicated in the figure legend. Reactions were terminated after 10 min by addition of an equal volume of 3.5% perchloric acid. After neutralization with $KHCO_3$, samples were analysed by HPLC using a LiChrosorb RP-18 column with 15% methanol, 0.1 M KH_2PO_4, 5 mM tributylamine, pH 6.4 as the mobile phase. Fractions were collected and radioactivity was determined.

G-protein activation and cAMP binding

Membranes were resuspended at a density of 4×10^8 cell equivalents/ml and incubated with ATPγS and 5 mM $MgCl_2$ in the presence or absence of cAMP or its analogues for 5 min at 20°C. Subsequently the membranes were washed three times in ice-cold PB to remove all additions. Binding of cAMP to surface receptors was measured at 0°C in a reaction (100 μl) containing 5 nM [^{3}H]cAMP, 5 mM dithiothreitol and membranes derived from 10^7 cells in the presence or absence of 10 μM GTPγS. After 1 min the incubation was centrifuged for 3 min at 14 000 *g*, the supernatant was aspirated and radioactivity in the sediment was determined. Non-specific binding was determined in the presence of 100 μM cAMP.

Activation of phospholipase C

Cells were resuspended to 5×10^7 cells/ml in 40 mM HEPES–NaOH pH 6.5 and lysed through nuclepore in the presence of 6.9 mM EGTA, 5 mM $MgCl_2$ and 10 μM ATPγS or 1 μM GTPγS as indicated. In case of cAMP stimulation, 10 μM 2′deoxy cAMP were applied 10 s after lysis. Samples were incubated for 1 min at 20°C to allow NDP kinase reactions. Subsequently phospholipase C was measured. Aliquots were taken and either quenched by the addition of an equal volume of 3.5% perchloric acid or incubated for 20 s at 10^{-5} M free Ca^{2+} before being quenched. Ins(1,4,5)P_3 levels in neutralized samples were determined using an isotope dilution assay (Van Haastert, 1989). Phospholipase C activity is determined from the amount of Ins(1,4,5)P_3 produced after raising the calcium concentration.

Inhibition of NDP kinase by monoclonal antibodies

Purified recombinant NDP kinase, 25 mg/ml, was incubated overnight at 4°C in 50 mM Tris–HCl pH 7.4 and 1 mg/ml BSA without antibodies, with the monoclonal antibody mAb8-6 or with the monoclonal antibody mAb9-7, both at 3 μM and assayed for NDP kinase activity as described (Kimura and Shimada, 1988).

Acknowledgements

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